Dear Dr. Sternberg:

I am pleased to accept your manuscript entitled "The <I>C. elegans</I> female state: Decoupling the transcriptomic effects of aging and sperm-status" for publication in G3, pending minor revision.

Please submit your revision along with a brief description of how you modified the manuscript in response to the reviewers’ concerns and suggestions (which can be viewed at at http://submit.g3journal.org). Please pay particular attention to the second reviewer's comment regarding additional experiments that could have been included involving mating to fertilization-defective males. This may be relevant given that factors present in seminal fluid in other systems (e.g. Drosophila) have been shown to influence female reproductive development and behavior even in the absence of functional sperm. In addition, please consider comments suggesting changes to terminology such as "epistasis" and the "female state", and whether modifying the usage of such descriptors could help clarify the narrative for readers. In your response please elaborate on the rationale for how you chose to respond to the reviewer's queries.

I expect you should be able to submit a revised manuscript within 30 days. A suitably revised manuscript will be acceptable for publication; I don’t expect to send it out for re-review.

Thank you for submitting this story to G3.

With best regards,

Kris Gunsalus  
Associate Editor

**Response to the Editor**

We have amended the text to reflect the reviewer comments. We have added a section briefly comparing our results with *Drosophila* behavioral changes induced by seminal fluid and amended our discussion of experiments that could have been included. We have also tried to make changes to terminology to make the text clearer by changing the term ‘female state’ to ‘female-like state’. We have removed

**Reviewer 1 Reviewer's Comments to Author...**

This manuscript describes a well-designed study, in which the effect of absence/depletion of sperm on gene expression can be distinguished from natural aging. The data provide updated identification of genes that are differentially expressed during aging, and point to small subset that are more strongly associated with the transition from self-fertile to self-sterile hermaphrodite. The figures and legends are appropriate and provide the necessary information to interpret the findings. The bioinformatics analysis is well-supported and carefully explained, and this report has the value of describing in detail a more sophisticated and accurate linear regression method to analyze a two-factor expression experiment that works much better than simple pairwise comparisons (which suffers from artifacts due to assumptions, cutoffs, etc). It’s not clear that a lot of new information about the biology of aging or sperm loss is learned from the resultant datasets, although it is possible that some hidden gems are present for those interested in follow-up studies.

**Major comment**

The authors should rewrite the second half of the abstract to avoid the confusion that comes from the reader not yet understanding how they did their study (ie did not perform pairwise comparisons). The statement about fog-2 mutants phenocopying d6 wt is confusing. Do the authors mean that d1 fog-2 mutants phenocopy d6 wt, and that’s why it’s epistatic? I think it’s also tricky to use the word epistatic here, w/r/t gene expression. The authors later state in the text that “when the two perturbations are loss-of-function mutations, such interactions are epistatic interactions” (p3, line 200). Thus, by the authors’ definition, this seems inappropriate as only one loss-of-function mutation is analyzed. However, even later in the manuscript, they explain their rationale more thoroughly. Regardless, it would ultimately be helpful to the reader to avoid the confusing vocabulary at this early stage of the manuscript.

Author Response:

We have amended the abstract to try to make it clearer. We have removed the term ‘phenocopying’ and replaced it with a non-jargon phrase. We have also explained the use of the term ‘epistasis’ in more depth when it comes up in the results section.

**Minor comments:**

How was significance for the TEA determined? At least a brief mention is warranted, given the low enrichment levels generally (e.g. 1.4x)

Significance in TEA is determined using a hypergeometric test. The hypergeometric test is extremely sensitive to deviations from a null distribution, so statistical significance is not necessarily correlated with impressive fold-changes. This is particularly true when the terms have hundreds of annotations, in which getting an enormous fold-change becomes difficult if not impossible. As a corollary to this, observing >20 fold-changes in an Ontology Enrichment Analysis often means that the term is expected to be rare because only a few genes are annotated to this.

There is a throwaway line in the Figure legend for Fig 3 about fog-2 having “more RNA” than wild type at both collection points. Why? Should be discussed.

We apologize for the confusion. Our point was not that one sample had “more mRNA” than the other at either collection point. Rather, we intended to say that *fog-2* had increased expression levels of the hypothetical gene relative to the wild type, which is reflected in a line with a shifted intercept in Figure 3A and 3B. In other words, this gene would be identified as a differentially expressed gene associated with loss of *fog-2*.

Line 264 – The authors state “terms like AB and midbody likely reflect the impact of fog-2(lf) on the germline”. This could be explained more – why these terms? AB refers to the AB blastomere, presumably. How can this be related to the germ line?

We would like to thank the reviewer for identifying an error in our writing. As written, the phrase made no sense. We checked our analyses and re-wrote the phrase to correct this error.

Line 320 – missing a reference

We have added the missing reference.

**Reviewer 2 Reviewer's Comments to Author...**

Review of: “The C. elegans female state: Decoupling the transcriptomic effects of aging and sperm-status”

Summary: In this manuscript, the authors dissect the relationship between time and reproductive status in aging C. elegans hermaphrodites. To do so, they compare the transcriptomes of 1- and 6-day old wildtype adults and fog-2 females. Their results indicate that many of the changes observed early in the aging process are a consequence of sperm depletion, rather than of aging per se. Their analysis also uses

sophisticated statistical methods to describe the transcriptional phenotypes of whole animals.

**Recommendation: Accept after minor revisions**

**Comments:**

(1) The use of the term ‘female state’ is misleading and must be eliminated. Although C. elegans XX animals that never produce sperm can be referred to as females as a way of comparing them to the state of their XX ancestors, this term may not be used for animals that have made sperm but then used all of them up. Perhaps the best approach would be to use ‘reproductively active’ for hermaphrodites with sperm, and ‘sperm depleted’ for hermaphrodites that no longer have sperm. This change would also provide a more natural rhetorical framework for the paper, since the conclusion would no longer be anticipated by the language chosen to describe the older hermaphrodites.

We have amended the name of the state to ‘female-like state’. We choose this nomenclature because spermless animals of either genotype in our study have identical characteristics. By using the term ‘female-like state’ we are making a statement about the physiology of the worm, not about its sex. The only exception to this is in the title.

(2) The authors claim on page 7 that it is not technically possible to determine if sperm introduced by males causes the same transcriptional state as those made by selfing hermaphrodites, I do not believe their arguments. Indeed, were this manuscript submitted to Genetics rather than G3, I would have insisted on precisely that experiment to resolve the scientific questions directly. This comparison could be done without initiating embryonic transcription by crossing fertilization-defective males with fog-2 L4 larvae, and either hand-picking the mated animals after 1 day, or using screens to separate the males from the mated females. Although such an experiment is not needed for acceptance by G3, the text must be changed to avoid stating that it is impossible.

We have amended the text to reflect this suggestion.

(3) The phrase “fog-2(lf) transcriptome” would seem to indicate all of the genes that are expressed in fog-2(lf) animals, but that does not appear to be how it is used here. Instead, the authors seem to be using it to mean all genes that differ in expression between fog-2 and the wild type. This must be clarified.

We have clarified the text to reflect this suggestion.

(4) On page 5, why would AB reflect germline? Does AB indicate a descendent of AB? Please explain.

The term that is pointed out was included in error and has been corrected (see response to reviewer 1).

(5) I am uncomfortable with the use of the term Phenotype enrichment of genes, since it implies there actually is a phenotypic difference. Is there a better alternative?

We have amended the text.

**Minor changes:**

Page 1: Change “Although C. elegans is traditionally thought of as a hermaphrodite, XX animals exhaust their sperm” to “Although C. elegans XX animals are hermaphrodites, they exhaust their sperm”

We have amended the text to incorporate this suggestion.

Page 3: Line 129 should not be indented

We have removed the indentation.

Page 5, line 286 Change “either aging or germline feminization: to “to either aging or germline feminization”

We have changed this line.

Page 5, line 316: Change “these genes define a female state” to “these genes define a spermless state”

See response to (1)

Page 5, line 320 “epistasis between genes in a pathway ().” Add citation.

We have added the citation.

Page 7, line 390 Change “promotes a non-female states,” to “promotes a non-female state,”

We have removed this line in re-writing the text.

Figure 2b: It would be helpful to show some non-enriched tissues for comparison.

TEA does not automatically plot non-enriched tissues, in part because the fold-change enrichment is a function of number of annotations. Terms that have many annotations will not exhibit large fold-changes, whereas terms that have a very small number of annotations will.